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3-Chloro-5-Substituted-1,2,4-Thiadiazoles (TDZs) as Selective and Efficient Protein Thiol Modifiers

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The study of cysteine modifications has gained much attention in recent years. This includes detailed investigations in the field of redox biology with focus on numerous redox derivatives like nitrosothiols, sulfenic acids, sulfinic acids and sulfonic acids resulting from increasing oxidation, S-lipidation, and perthiols. For these studies selective and rapid blocking of free protein thiols is required to prevent disulfide rearrangement. In our attempt to find new inhibitors of human histone deacetylase 8 (HDAC8) we discovered 5-sulfonyl and 5-sulfinyl substituted 1,2,4-thiadiazoles (TDZ), which surprisingly show an outstanding reactivity against thiols in aqueous solution. Encouraged by these observations we investigated the mechanism of action in detail and show that these compounds react more specifically and faster than commonly used *N*-ethyl maleimide, making them superior alternatives for efficient blocking of free thiols in proteins. We show that 5-sulfonyl-TDZ can be readily applied in commonly used biotin switch assays. Using the example of human HDAC8, we demonstrate that cysteine modification by a 5-sulfonyl-TDZ is easily measurable using quantitative HPLC/ESI-QTOF-MS/MS, and allows for the simultaneous measurement of the modification kinetics of seven solvent-accessible cysteines in HDAC8.

Introduction

Cysteines are underrepresented amino acids in proteins, which are very often highly conserved in protein families indicating their structural and functional importance. Cysteines can undergo numerous posttranslational modifications (PTMs), which maintain redox homeostasis inside cells, as well as cell signaling via coupled redox systems.[1] There is a range of reversible thiol modifications, such as intramolecular or intermolecular disulfide formation between proteins, but also between a protein and a low molecular weight thiol. Other modifications include nitrosothiols, sulfenic acids, sulfinic acids and sulfonic acids resulting from increasing oxidation, S-lipidation, and perthiols.[1c,2] All of these modifications have been shown to be involved in a variety of biological processes.[3] Thus, there is a growing interest in analyzing these PTMs to expand the knowledge about the biological role of a cysteine containing protein in every possible modification state and correlate these results with biological activity. The typical chemical approach to study cysteine modifications on proteins involves selective labeling of reduced cysteine residues by a highly reactive blocking agent, which is compatible with aqueous solutions.[2a,4] Effective capture of unmodified cysteines prevents subsequent unspecific oxidation or other thiol-mediated cross-reactions, which can obscure the analysis of cysteine modifications during sample preparation. Chemical modification of cysteines are widely used to facilitate biophysical studies for the elucidation of molecular mechanisms.[5]

Furthermore, originally unmodified cysteines can be unambiguously identified by mass spectrometry and the expected mass shift upon thiol blocking. If more than one cysteine is contained in an analyzed peptide fragment, the specific position, where the modification has occurred, can be assigned by using MS/MS fragmentation procedures.[6] Most standard operation procedures employ iodoacetamide (IAM) or *N*-ethyl maleimide (NEM) as rather thiol-selective thiol-modifier in the first step of cysteine PTM analysis. Consequently, the efficiency of assays to examine thiol-modifications relies heavily on the reactivity and selectivity of the used thiol modifying reagent. Although widely used, IAM and NEM have been shown to also react with other nucleophilic amino acids, such as lysine or histidine.[7] Consequently, thiol blocking reagents with improved reactivity and selectivity are required to optimize the analysis of protein PTMs at cysteine residues, which is particularly true for large scale proteomic studies. Methylsulfonyl benzothiazole (MSBT) has been suggested as effective and selective thiol blocker alternative to commonly used IAM and NEM.[8] MSBT contains a methylsulfonyl leaving group,

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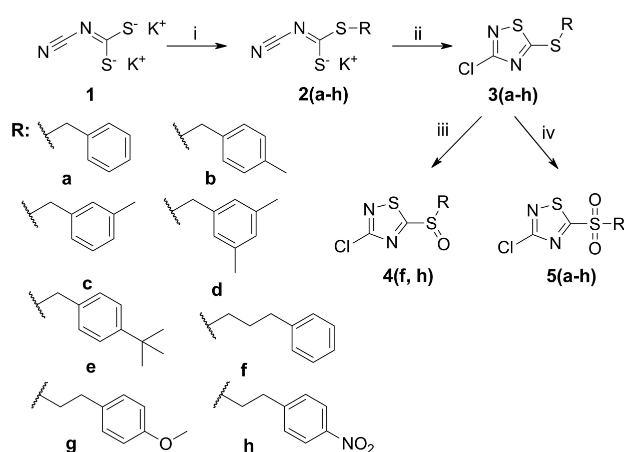
which is attached to the C-2 position of a 1,3-benzothiazole moiety. Interestingly, we identified very similar compounds as inhibitors of human histone deacetylase 8 (HDAC8), and wondered, whether these compounds unfold their inhibitory effect through modification of C153, which is located in the active site pocket of HDAC8. In this study we elucidate the molecular interaction between thiol groups in HDAC8 and low molecular weight thiol model compounds with 3-chloro-1,2,4-thiadiazole (TDZ) analogues, which carry organo-sulfonyl, -sulfinyl or –sulfanyl substituents at 5-position as potential leaving groups. The observed thiol-modifications are further analyzed by kinetic experiments, quantitative HPLC/ESI-QTOF tandem mass spectrometry and supporting techniques.

Results and Discussion

In our current research attempts we discovered a variety of 5-sulfonyl-3-chloro-1,2,4-thiadiazoles as potent HDAC8 inhibitors. To explore the structure activity relationship and the mode of action of this class of compounds further, we synthesized a series of analogues. The synthesis of 3-chloro-5-substituted-1,2,4-thiadiazoles was performed according to Wittenbrook (Scheme 1).[9] Dipotassium cyanodithioimidocarbonate (**1**) was alkylated with various alkyl bromides (**a**–**h**) to give the corresponding monoalkyl derivatives **2**(**a**–**h**). 3-chloro-5-alkylsulfanyl-1,2,4-thiadiazoles **3**(**a**–**h**) were synthesized by oxidative cyclization of monoalkyl derivatives **2**(**a**–**h**) with sulfuryl chloride. The obtained sulfanyl compounds **3**(**a**–**h**) were oxidized to sulfinyl compounds **4**(**f**, **h**), and sulfonyl compounds **5**(**a**–**h**) using *in situ* generated peracetic acid (Scheme 1):

The IC_{50}-values of **5(a—h)** were very similar between 0.1 and 0.3 μM under assay conditions suggesting that the R-group has essentially no influence on the inhibitory activity (Table S4). Corresponding compounds **4f** and **4h** were similarly active. All tested compounds of the **4**- and **5**-series showed high selectivity (20–160 fold) for class I HDAC HDAC8 compared to class IIa member HDAC4. This might be explained by the fast modification of cysteines C153 and C275, which are involved in redox-regulation of HDAC8.[10] The cysteine redox switch partner of C153 in HDAC8, C102, as well as C275 are not present in HDAC4. In comparison, **3(a—h)** showed significantly lower inhibitory activity against HDAC8. Therefore, the SO_2- and SO-functionalities turned out as determining factor for HDAC8 activity.



Scheme 1. Synthesis of 2-chloro-5-substituted thiadiazole derivatives: i) RBr (**a**–**h**), H_2O, acetone, 0 °C, r.t., 24 h, yield 55–88 %; ii) SO_2Cl_2, $CHCl_3$, 0 °C, r.t., 24 h, yield 43–78 %; iii) 1.2 eq 30 % H_2O_2 (aq), 0 °C, r.t., 24 h, 25–90 %; iv) 3 eq 30 % H_2O_2 (aq), 0 °C, r.t., 24 h, yield 31–87 %.

Based on our knowledge of the special disulfide and cysteine dependent regulation mechanism of this enzyme and the structural similarity to the published cysteine modifier MSBT, we wondered whether compounds from the **4**- and **5**-series unfold their inhibitory potential through cysteine thiol modification. To test the general reactivity against thiols we used the thiol reactivity assay first described by Resnick et. al.[11] For that purpose we followed the time dependent loss in light absorption at 412 nm of 5-thio-2-nitrobenzoic acid (TNB^{2-}) generated with TCEP from 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) upon reaction of a cysteine modifier (Figure 1A and B). To our surprise we figured out that the reaction of 5-substituted sulfonyl TDZ (**5h**, 5-SO_2-TDZ) occurs nearly immediately after mixing TNB^{2-} with a stoichiometric amount of cysteine modifier (Figure 1B). The reaction was so fast that we were not able to observe a time dependent decrease in absorption in the time window of our instrumentation.

On the other hand, structurally very similar 5-substituted sulfinyl TDZ (**4f**, 5-SO-TDZ) is less reactive than the sulfonyl analogue. For this compound we observed a decrease in absorption in the first minutes of measurement. Interestingly, the 5-substituted sulfanyl analogue (**3h**, 5-S-TDZ) shows no reactivity towards free thiols. As a control we followed the reaction of *N*-ethylmaleimide (NEM) with TNB^{2-} under the same experimental conditions. NEM reacts much slower than the examined **5h** and **4f**. All thiol-reactive compounds react to nearly 100 % completion. These first experiments let us conclude, that TDZs are highly thiol reactive compounds with much higher reactivity against TNB^{2-} as the frequently employed cysteine modifier NEM. A general reaction scheme for sulfonyl, sulfinyl and sulfanyl TDZ analogues is shown in Figure 2. The next question we asked was, whether TDZs are reacting solely with cysteine or also with other nucleophilic amino acids like serine, histidine and lysine. For that we set up several reactions and analyzed the conversion of the used 5-sulfonyl, sulfinyl and sulfanyl substituted TDZs by thin layer chromatography (TLC), HPLC-MS and HRMS.

The results (Figures S1–S19) show that the probed **5h** is not solely reacting with cysteine. It also consumes all of the histidine after 1 h at 30 °C. In contrast, the slower reacting **4f** basically reacts only with cysteine (Figures S2, S5–S13). We confirmed that **3h** (5-S-TDZ) is unreactive against cysteine and also does not react with histidine, lysine or serine (Figures S15–S17). Therefore, **4f** modifies cysteine selectively with a slightly lower reactivity compared with **5h**. Nevertheless, the reactivity of the probed **4f** is still much higher than that of the commonly used NEM, which makes it in our opinion an excellent agent for selective and rapid thiol blocking in aqueous solutions.

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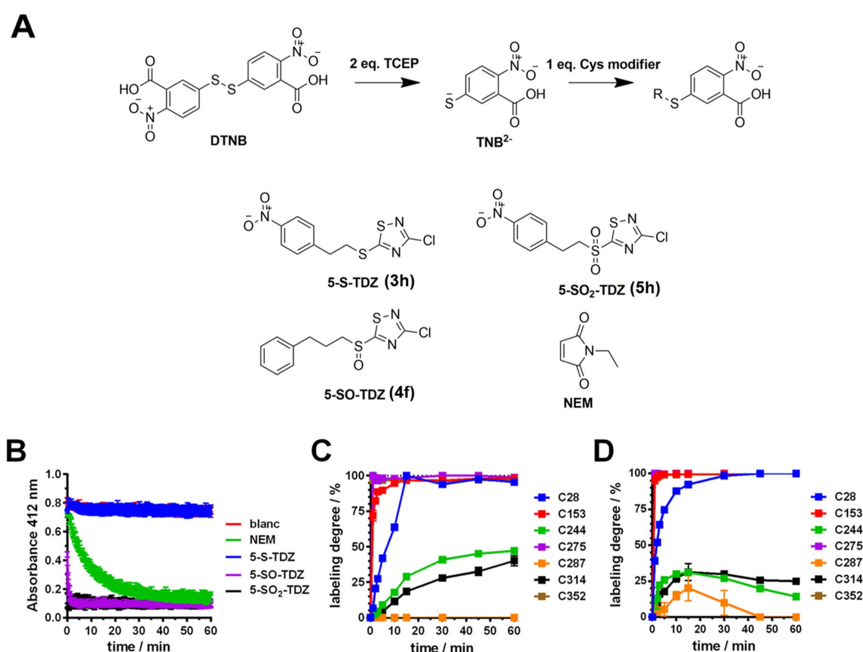

Figure 1. TNB^{2-} reactivity assay and covalent HDAC8 labelling. A) is showing the general reaction scheme of the used TNB^{2-} reactivity assay and the structures of the used compounds as cysteine modifiers. B) Reactivity of the four studied cysteine modifier against TNB2- followed by decrease in absorbance at 412 nm. Time dependent increase in labeling degree of HDAC8 bound cysteines for *N*-benzyl maleimide (C) and **5h** (D), as determined from quantitative HPLC/ESI-QTOF measurements

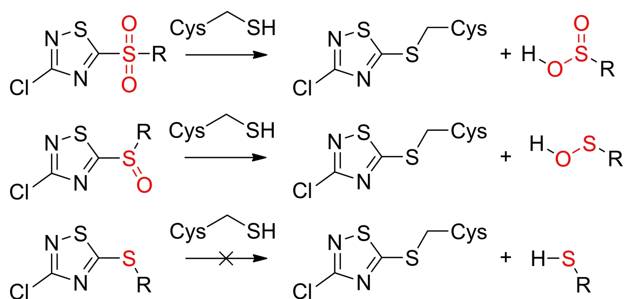

Figure 2. Reaction scheme for the reaction of 5-SO_2-TDZs, 5-SO-TDZs and 5-S-TDZs with free cysteine thiols. 5-SO_2-TDZs are reacting faster than 5-SO-TDZs, whilst 5-S-TDZs are showing no reactivity against free thiols.

Encouraged by the fast reactivity and selectivity towards cysteine we tested the potential for labeling of cysteine residues in HDAC8. HDAC8 consists of 10 cysteines showing special redox regulative features.[10] We quantified the degree of labeling for seven solvent accessible cysteine residues of HDAC8 by tryptic digestion and high resolution HPLC-MS/MS. With this approach we investigated whether **5h** has a beneficial labeling behavior by means of reactivity compared with *N*-benzyl maleimide. The protein was incubated with a 40-fold molar excess of arylating agent. The reaction was stopped after different indicated reaction times by trichloroacetic acid mediated protein precipitation. After removing cysteine modifiers in the supernatant, remaining free thiol groups of precipitated protein were labelled using NEM to enable the precise quantification of thiol groups not modified by **5h** or *N*-benzyl maleimide during the respective reaction time period. The amount of *N*-benzyl maleimide and TDZ-modified peptide fragment was normalized to that of NEM modified peptide fragment. The amount of alkylated fragments was calculated by the sum of the areas of all charge states of the respective peptide fragments. The exact theoretical and observed masses of each single alkylated cysteine containing fragment are summarized in the supporting information (Table SI 1–3). The peptide fragment containing cysteines 102, 125 and 131 was not analyzed because all three cysteines are part of one fragment and therefore, we were not able to determine, which cysteine exactly was alkylated at each time point of alkylation. In the case of no reaction with the specific modifier the amount of NEM labelled peptide fragment was 100% and decreased with increasing amount of specific labeled peptide fragment up to 100% labeling degree with increasing modification by *N*-benzyl maleimide or **5h**. Like for our observations for the reaction of **5h** with TNB^{2-}, **5h** reacts faster with cysteine thiols in protein context than the control compound *N*-benzyl maleimide. In our opinion this makes **5h** an outstanding protein thiol blocker for several applications in the field of cysteine modifications and cysteine dependent redox biology, where fast blocking of nascent thiols is mandatory to freeze specific redox states and prevent disulfide shuffling. If specificity towards cysteine is needed, we recommend using the slower but still fast, reacting 5-SO-analogues. To test for a potential biological application we performed a commonly used assay in the field of redox biology, the biotin switch assay, and checked whether our described thiol blocker is capable of blocking free or nascent thiols.[12] At first, we labeled HDAC8 with **5h** and NEM. Afterwards, we labeled free thiols with *N*-biotin maleimide

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and conducted a western blot and quantified free thiol content with an HRP-Streptavidin conjugate. As expected, the probed **5h** and **4f** analogues show comparable results to NEM as control (Figure 3).

The 5-SO-TDZ and 5-SO_2-TDZ scaffolds add to the repertoire of already known heteroaryl sulfones reacting by nucleophilic aromatic substitution reactions (SnAr).[13]

Retrospective to our first experiments and the observed inhibitory potential against HDAC8, we hypothesize that the new scaffolds may be useful as integral part of enzyme inactivators. However, it is anticipated, that a rational design of such covalent inactivators would be challenging, because specific non-covalent recognition by the target protein has to be optimized by rational design of both, a suitable substitution pattern at the TDZ-ring, and the structure of the leaving group containing the sulfonyl- or sulfinyl-group. Furthermore, it is of utmost importance to reduce the reactivity of optimized inactivators by design in order to prevent undesired off-target effects. For example, changing the substituent in the 2 position of the TDZ ring should allow to tune the cysteine-reactivity of this class of inactivators. Despite these challenges, integral TDZ-groups with suitable leaving groups might offer new opportunities in the design of covalent inactivators apart from known and partly clinically approved covalent inhibitors, which are limited to acrylamide, haloacetamide or maleimide functionalities that are usually later connected to an existing non-covalent inhibitor.

Conclusion

In summary, this study describes for the first time 5-SO_2- and 5-SO-TDZs as promising cysteine reactive chemicals with the potential for versatile usage in biorthogonal tagging, classic biotin switch assays, but also in MS-based proteomic studies. 5-SO_2-TDZ or 5-SO-TDZ analogues are anticipated to serve as beneficial blocking reagents in the field of redox biology because of their fast cysteine modifying reaction kinetics in aqueous environment. Also, TDZ analogues with tunable cysteine reactivity and carefully designed substitution patterns may be useful in the design of specific covalent inactivator compound.

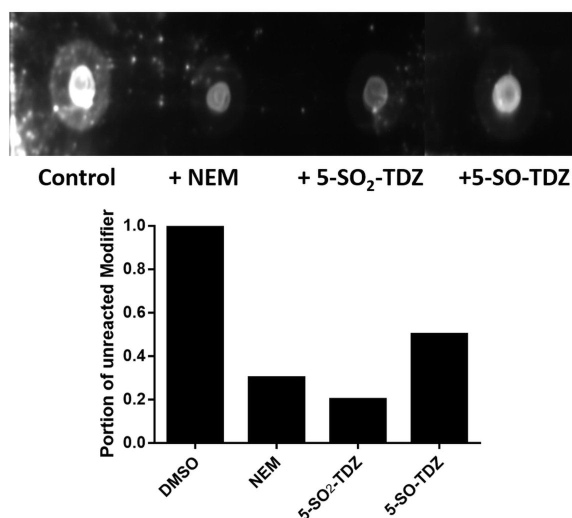

Figure 3. Dot Blot for cysteine reactivity of different TDZs with HDAC8. HDAC8 cysteines were blocked with indicated TDZs and *N*-ethylmaleimide (NEM) as a common thiol blocking agent. A DMSO control without thiol blocking agent was also prepared. Unblocked thiols were subsequently blocked with NEM-biotin. Brightness of dots indicate HRP activity of Streptavidin-HRP conjugate bound to biotin labeled thiols.

Experimental Section

HDAC8 production

HDAC8 was produced and purified as described earlier according to Jänsch et al.[10b]

Synthesis

General procedure for the synthesis of **2**(**a**–**h**): To a stirred solution of dipotassium cyanodithioimidocarbonate (**1**) (500 mg, 2.57 mmol) in water (2 mL) and acetone (2.20 mL) previously cooled to 0 °C is added in three portions over three hours a solution of appropriate arylalkyl bromide (2.57 mmol) in acetone (1.5 mL). The reaction mixture is allowed to stir overnight at room temperature. The vigorous stirring is maintained throughout the reaction time. The reaction is monitored by thin-layer chromatography (the first eluent chloroform was used to determine unreacted arylalkyl bromide, the second eluent ethyl acetate containing 2% ethanol is used to monitor the formation of the product). Solvents are removed under reduced pressure raising the water bath's temperature evenly from 45 °C to 60 °C. A solid residue is slurred in acetone, the inorganic salt is filtered off, the filtrate is concentrated under reduced pressure to furnish a solid residue which is treated with ethyl acetate. The resulting mixture is filtered to remove insoluble impurities. Ethyl acetate is removed under reduced pressure, a solid residue is suspended in chloroform, stirred for one hour, filtered and washed with chloroform.

General procedure for the synthesis of **3**(**a**–**h**): To a stirred suspension of the appropriate compound **2**(**a**–**h**) (1.06 mmol) in chloroform (1.6 mL) previously cooled to 0 °C sulfuryl chloride (1.28 mmol) is added in three portions every 30 minutes. The reaction mixture is allowed to stir overnight at room temperature. The progress of reaction is monitored by TLC (first eluent chloroform is used to monitor the formation of the reaction product, then ethyl acetate with 2% ethanol to check that all of the starting compound has reacted). The solvent is evaporated under reduced pressure. The product is purified by vacuum chromatography on a column of silica gel.

General procedure for the synthesis of **4**(**f**, **h**):To a stirred solution of the appropriate compound **3**(**f**, **h**) (0.500 mmol) in a mixture of acetic acid (250 μL) and acetic anhydride (250 μL) previously cooled to 0 °C is added 30% H_2O_2(aq.) (0.600 mmol, 0.118 mL) in three portions every hour. The reaction mixture is allowed to stir overnight at room temperature. The reaction progress is monitored by TLC (hexane/chloroform 2:1). The solvents are removed under reduced pressure and the product is purified by vacuum chromatography.

General procedure for the synthesis of **5**(**a**–**h**): To a stirred solution of the appropriate compound **3**(**a**–**h**) (0.500 mmol) in a mixture of acetic acid (250 μL) and acetic anhydride (250 μL) previously cooled

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to 0 °C is added 30% H_2O_2(aq.) (1.50 mmol, 0.148 mL) in three portions every hour. The reaction mixture is allowed to stir overnight at room temperature. The reaction progress is monitored by TLC (hexane/chloroform 2:1). The solvents are removed under reduced pressure and the product is purified by vacuum chromatography. The details of the characterization of the new compounds are reported in Supporting information.

TNB^{2-} reactivity assay

To test reactivity of cysteine modification 100 μM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was reduced with 200 μM tris(2-carboxyethyl) phosphine (TCEP) to yield 200 μM 5-thio-2-nitrobenzoic acid (TNB^{2-}). Reaction was started by adding 200 μM cysteine modifier in sodium phosphate buffer (20 mM, pH 7.4) with 150 mM NaCl and decrease in absorption at 405 nm was monitored using a Tecan Spark plate reader.

Thin layer chromatography

Protocols and methods for conducting and quantification of thin layer chromatography data are provided in the supplementary information.

Protein labelling

HDAC8 was labelled for subsequent digestion and mass analysis by mixing 1 mg/mL protein with 1 mM benzyl-maleimide or 5-SO_2-TDZ in sodium phosphate buffer (20 mM, pH 7.4) with 150 mM NaCl in a final volume of 100 μL. Reactions were stopped, and protein was precipitated by adding 10 μL trichloroacetic acid (TCA, 100%). Afterwards, samples were centrifuged at 18000 g for 5 mins and supernatant was discarded. The remaining pellet was than dissolved in 100 μL alkylation buffer (6 M Gdn-HCl, 50 mM NEM, 10 mM TCEP, 100 mM sodium phosphate, pH 8.0) and mixed slightly for 30 minutes at 30 °C. After alkylation 900 μL ice-cold ethanol was added to precipitate protein again. HDAC8 immediately precipitates and the turbidity raises. After 30 minutes in the freezer at −20 °C the samples were centrifuged at 18000 g and 4 °C for 15 minutes and again the supernatant was discarded. Finally, the precipitate was dried with an open cap for about 30 minutes at 30 °C.

Tryptic digestion

HDAC8 precipitates were dissolved in 10 mM NH_4HCO_3 pH 8 to get a protein solution with a concentration of 1 $mg*mL^{-1}$ HDAC8. Tryptic digestion was performed with 1 μg/mL trypsin over night while shaking at room temperature. The digestion process was stopped by freezing and storing the samples at −20 °C until measurement.

HPLC-MS/MS

The separation of peptides was performed using the Shimadzu ExionLC AD UHPLC System with a ZORBAX 300SB-C18, 1.8 μm, 2.1× 100 mm column which was directly coupled to an X500R QTOF mass spectrometer (AB Sciex) equipped with an IonDrive Turbo V ESI-source. After injection of 4 μL sample onto the column, separation was carried out under gradient conditions of the solvents ACN (A) and water (B) with 0.1% FA each at a flow rate of 0.2 mL/min. The separation started with 10% A for 1 min and an increase to 25% A in 3 min. It was followed by a slow linear gradient to 40% A in 11 min, hold for 1 min, and a fast linear gradient to 90% A in 1 min, which was hold for 2 min. To reach starting conditions again, a linear gradient to 10% A in 2 min was performed and 10% A was hold for 3 min. The parameters used for ESI-QTOF-MS/MS analysis in positive mode were as follows: nebulizer (gas 1), 55 psi; turbo gas (gas 2), 55 psi; curtain gas, 35 arbitrary units; collision gas (CAD gas), 7 arbitrary units; source temperature, 450 °C; ionspray voltage, +5 kV; declustering potential (DP), 20 V; collision energy (CE), 10 V. TOF MS full scan and product ion Data were acquired by information dependent acquisition (IDA) scan mode in the mass range of 300–2000 Da. Dynamic collision energy mode was set active. The software BioPharmaView was used to analyze the mass spectrometric data. The expected mass differences after modification of cysteines with *N*-ethylmaleimide (125,048 Da), 3-chloro-1,2,4-thiadiazole (117,939 Da) and *N*-benzylmaleimide (187,063 Da) were set. The modifications could replace disulfide bridges and the protein was present in reduced form. For *in silico* hydrolysis, trypsin as used enzyme, three maximum modifications per peptide and a maximum of one missed enzyme site was set. The m/z tolerance for matching a measured peptide to a theoretical peptide was ±5 ppm and the tolerance for successful assignment of fragment ions was ±0.03 Da. Assigned peptides must achieve a score of at least 3.0 to be included in the calculation of the fraction of modified cysteine-containing peptides. The score is based on the accuracy of the assignment of a precursor ion and the associated fragment ions and can take on values between 0 and 100. The higher the score, the more reliable the assignment of the respective peptide. For the semiquantitative analysis of the fraction of covalently modified cysteines, the peak areas (XIC area) of the cysteine-containing peptides successfully assigned with a score of at least 3.0 were used from the extracted ion chromatogram (XIC). The calculation of the fraction of covalently modified cysteines was performed individually for each cysteine-containing peptide according to Equation 1.

$$\frac{\sum XIC\ area\ (peptide\ X\ with\ modification)}{\sum XIC\ area\ (peptide\ X\ with\ and\ without\ modifications)} * 100\ \% \qquad (1)$$

Biotin-switch assay

A solution of 23.8 μM HDAC8 and 953 μM modifier was prepared in assay buffer (25 mM Tris-HCl, pH 8.0, 75 mM KCl, 0.001% Pluronic F-127) and was incubated for 1 h at 30 °C and 450 rpm. Subsequently 10% trichloroacetic acid was added to the solution and incubated for 10 min at room temperature under occasionally mixing to precipitate the protein. The solution was then centrifuged for 15 min. The supernatant was discarded and the precipitate resuspended in alkylation buffer (6 M Guanidine Hydrochloride, 1 mM *N*-Ethylmaleimide-Biotin, 10 mM Tris-(2-carboxyethyl)-phosphine, 100 mM Na3PO4, pH 8.0). The solution was incubated for 1 h at 30 °C and 450 rpm. For precipitation, the solution was diluted in a 1:10 ratio with −80 °C absolute ethanol and stored at -80 °C for 2 h. Afterwards the sample was centrifuged for 15 min and the pellet was washed twice by the addition of fresh absolute ethanol, centrifugation and discarding of the supernatant and left to dry for 1 h. Subsequently, the precipitate was resuspended in alkylation buffer without *N*-Ethylmaleimide-Biotin. 2 μL of sample was directly applied on a nitrocellulose blotting membrane (Nitrocellulose Blotting Membrane, Amersham Protran 0.2 μm NC, GE Healthcare Life Science, Germany) and left to dry for 1 h at room temperature. The membrane was treated with blocking buffer (5% BSA in TBS-T buffer) for 1 h at 4 °C. After discarding the blocking buffer, the membrane was incubated with a 1:10000 diluted horseradish peroxidase-streptavidin conjugate (HRP-Conjugated Streptavidin, Thermo Scientific, Germany) in TBS-T overnight at 4 °C. To remove the HRP-conjugate the membrane was washed with

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TBS-T buffer, once for 15 min and twice for 5 min. Finally, the membrane was washed once for 5 min with TBS buffer. For measuring, 300 μL of HRP substrate was prepared (Luminol Enhancer Solution, Amersham ECL Prime and Peroxide Solution; GE Healthcare UK Limited, Little Chalfont Buckinghamshire; 1:1 ratio) and added to the membrane. The chemiluminescent signal was captured with a CCD camera (Celvin S, Biostep GmbH, Burkhardtsdorf, Germany).

All centrifugation steps were carried out at 18000 g and 4 °C. TBS buffer contained 25 mM Tris-HCl, 150 mM NaCl at pH 7.5. TBS-T buffer was TBS buffer with 0.05% Tween 20 (VWR Chemicals, Radnor, PA, USA).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biotin switch assay · covalent inactivators · nucleophilic aromatic substitution · proteomic studies · thiol modification

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